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In Vitro Synergy of Colistin Combinations against Colistin-Resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* Isolates

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Colistin resistance, although uncommon, is increasingly being reported among Gram-negative clinical pathogens, and an understanding of its impact on the activity of antimicrobials is now evolving. We evaluated the potential for synergy of colistin plus trimethoprim, trimethoprim-sulfamethoxazole (1/19 ratio), or vancomycin against 12 isolates of *Acinetobacter baumannii* ($n = 4$), *Pseudomonas aeruginosa* ($n = 4$), and *Klebsiella pneumoniae* ($n = 4$). The strains included six multidrug-resistant clinical isolates, *K. pneumoniae* ATCC 700603, *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and their colistin-resistant derivatives (KPm1, ABm1, and PAm1, respectively). Antimicrobial susceptibilities were assessed by broth microdilution and population analysis profiles. The potential for synergy of colistin combinations was evaluated using a checkerboard assay, as well as static time-kill experiments at 0.5× and 0.25× MIC. The MIC ranges of vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole (1/19) were ≥128, 4 to ≥128, and 2/38 to >128/2,432 μg/ml, respectively. Colistin resistance demonstrated little impact on vancomycin, trimethoprim, or trimethoprim-sulfamethoxazole MIC values. Isolates with subpopulations heterogeneously resistant to colistin were observed to various degrees in all tested isolates. In time-kill assays, all tested combinations were synergistic against KPm1 at 0.25× MIC and 0.5× MIC and ABm1 and PAm1 at 0.5× MIC. In contrast, none of the tested combinations demonstrated synergy against any colistin-susceptible *P. aeruginosa* isolates and clinical strains of *K. pneumoniae* isolates. Only colistin plus trimethoprim or trimethoprim-sulfamethoxazole was synergistic and bactericidal at 0.5× MIC against *K. pneumoniae* ATCC 700603. Colistin resistance seems to promote the *in vitro* activity of unconventional colistin combinations. Additional experiments are warranted to understand the clinical significance of these observations.

Because of the rapid spread of antimicrobial resistance and the slow development of novel antimicrobials, Gram-negative infections are becoming very challenging for clinicians and a real threat to international public health (20). Gram-negative bacteria are characterized by the presence of an outer membrane, limiting the penetration of hydrophobic and/or large antibiotics. The protective function of the outer membrane mainly relies on the presence of lipopolysaccharide (LPS) constituents at the surface of the cell. Thus, studies investigating bacterial mutants of *Escherichia coli* producing defective LPS demonstrated their increased susceptibility to hydrophobic antibacterial agents and suggested greater penetration of the agents through the outer membrane (24). Colistin sulfate (also referred to as polymyxin E) is a cyclic polypeptide exhibiting detergent-like properties. Colistin is known to interact with LPS and phospholipids present at the surface of the outer membrane, to disturb membrane permeability, and finally to bind to phospholipids present at the surface of the cytoplasmic membrane. The last interaction is thought to result in disruption of the osmotic equilibrium and leakage of the cell contents (7, 10, 22). Increased permeability of the outer membrane secondary to colistin exposure should lead to increased permeability to hydrophobic and/or large molecules. A few studies have evaluated the potential for synergy of unconventional colistin combinations (13, 25). In contrast, little is known regarding the potential for synergy of colistin combinations against colistin-resistant bacillus isolates (3, 5, 18), and to date, no data are available on colistin-resistant *Klebsiella* spp., an emerging threat, considering the worldwide increased prevalence of carbapenemase-producing *Klebsiella pneumoniae* (2, 6, 23).

The objectives of our study were to evaluate the potential for synergy and bactericidal activity of colistin plus vancomycin, trimethoprim, or trimethoprim-sulfamethoxazole (1/19 ratio) against colistin-susceptible and -resistant strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *K. pneumoniae*. Observations made through this study were also expected to provide additional insights into the mechanism(s) of resistance to colistin among three major Gram-negative species.

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MATERIALS AND METHODS

Bacterial strains and media. Six clinical isolates of *A. baumannii* (AB1 and AB2), *P. aeruginosa* (PA4 and PA5), and extended-spectrum beta-lactamase-producing strains of *K. pneumoniae* (KP3 and KP4) were selected from the ABC Platform Bugs Bank Collection (ABC Platform, Université de Lorraine, Nancy, France). Three ATCC strains (*A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853; Fisher Scientific, SAS, Illkirch, France) and their colistin-resistant derivatives (ABm1, KPm1, and PAm1) were also included in our study.

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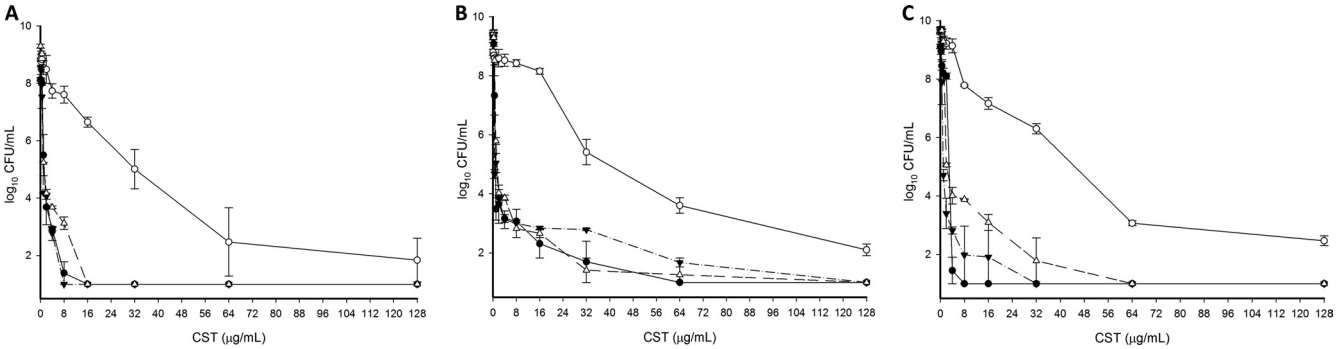

FIG 1 Colistin population analysis profiles. (A) ●, *A. baumannii* ATCC 19606; ○, ABm1; ▽, AB1; △, AB2. (B) ●, *K. pneumoniae* ATCC 700603; ○, KPm1; ▽, KP3; △, KP4. (C) ●, *P. aeruginosa* ATCC 27853; ○, PAm1; ▽, PA4; △, PA5. The error bars indicate standard deviations.

Strains ABm1 and KPm1 were selected *in vitro* using the gradient plate method, as previously described by Bryson and Szybalski (6a), whereas PAm1 was obtained from successive exposures to increased colistin concentrations, as described elsewhere (12). All three mutants were stable over 5 passages on drug-free agar. Mueller-Hinton broth II (MHB II) (Difco, Fisher Scientific, SAS, Illkirch, France) containing 12.5 μg/ml magnesium and 25 μg/ml calcium (supplemented MHB II [SMHBII]) was used for all *in vitro* experiments. The suitability of the medium for trimethoprim-sulfamethoxazole testing was verified using control strains according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (9). Mueller-Hinton agar (MHA) (Difco, Fisher Scientific, SAS, Illkirch, France) was utilized for growth and colony quantification.

Antimicrobial agents. Colistin sulfate, vancomycin, trimethoprim, and sulfamethoxazole were commercially obtained (Sigma-Aldrich, Saint Quentin Fallavier, France). Each agent was freshly prepared according to the CLSI guidelines in the appropriate solvent (9).

Susceptibility testing. MIC values were determined in duplicate according to CLSI guidelines at ~5.5×10^5 CFU/ml in SMHB II (9).

Colistin population analysis profiles (PAPs). The presence of subpopulations resistant to colistin was evaluated for all ATCC isolates, as previously described by Li et al. (19). Briefly, 50 μl of full 24-h cultures (10^9 CFU/ml) or appropriate serial dilutions in cold and sterile normal saline were plated onto MHA plates containing 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 μg/ml of colistin sulfate. After a 48-h incubation at 35°C, colonies were counted and $\log_{10}$ CFU/ml values were plotted versus time. Graphs were then used to calculate an area under the curve from zero to infinity ($AUC_{0-\infty}$) (SigmaPlot version 11.0; Systat Software). A heterogeneous population in terms of colistin resistance was defined as an isolate with detectable subpopulations growing in the presence of concentrations of colistin greater than the MIC, as defined by broth microdilution (19).

Bacteriostatic activity. Interactions between colistin and vancomycin, trimethoprim, or trimethoprim-sulfamethoxazole (1/19) were evaluated by a checkerboard titration assay in microplates, as described previously (11). Bacterial inocula were prepared as recommended for MIC determination. Colistin was tested at concentrations ranging from 1 to 64 and from 0.06 to 8 μg/ml for resistant and susceptible isolates, respectively. Vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole (1/19) were tested at concentrations varying from 0.25 to 128 μg/ml. Bacterial growth was visually assessed after 24 h of incubation at 35°C. The activity of each antimicrobial combination was estimated using the point of maximal effectiveness or index of fractional inhibitory concentration (ΣFIC). ΣFIC values were interpreted according to the following criterion: the potential for bacteriostatic effect, meaning the potential for synergy when ΣFIC is ≤0.5 (11). All experiments were performed in triplicate to ensure reproducibility, and the results were expressed as the mode values.

Synergy and bactericidal activity. The potential for synergy and/or bactericidal activity of colistin plus vancomycin, trimethoprim, or trimethoprim-sulfamethoxazole (1/19) was evaluated using a 2-ml time-kill assay and a starting inoculum of ~10^5 to 10^6 CFU/ml. Each experiment was performed in duplicate to ensure reproducibility. Antimicrobial regimens consisted of multiples of the MIC (0.25× and 0.5× MIC) of each agent alone or in combination. When the MIC value was greater than 128 μg/ml, we considered 128 μg/ml the MIC value. Samples were removed at 0, 1, 2, 4, 8, and 24 h. For all time-kill experiments, aliquots (100 μl) were serially diluted in cold and sterile normal saline. Bacterial counts were determined by plating three spots of 10 μl of appropriate dilutions on MHA plates and incubating them at 35°C for 18 to 24 h. Time-kill curves were then constructed by plotting mean colony counts ($\log_{10}$ CFU/ml) versus time. According to the 2012 *Antimicrobial Agents and Chemotherapy* Instructions to Authors, synergy was defined as a ≥2 $\log_{10}$ CFU/ml decrease between the combination and the most efficient agent alone at 24 h. The number of surviving organisms in the presence of the combination was also ≥2 $\log_{10}$ CFU/ml, and at least one of the drugs alone did not affect the growth curve of the tested organism. The bactericidal activities of drug combinations were defined as a ≥3 $\log_{10}$ CFU/ml (99.9%) reduction compared to the most active drug at 24 h.

RESULTS

Selection and characterization of colistin sulfate-resistant derivatives. Using subinhibitory concentrations, colistin-resistant strains of *A. baumannii, K. pneumoniae*, and *P. aeruginosa* ATCC isolates (i.e., ABm1, KPm1, and PAm1, respectively) were selected *in vitro*. All tested isolates appeared to be heterogeneously resistant to colistin, with the presence of subpopulations that grew in the presence of colistin concentrations higher than the MIC. However, colistin-resistant strains displayed a significant shift toward higher MIC values and exhibited greater proportions of subpopulations with higher colistin MICs (Fig. 1A to C). Of note, $AUC_{0-\infty}$ values ranged from 145.54 to 255.64 for colistin-susceptible isolates. In contrast, the colistin-resistant derivatives KPm1, ABm1, and PAm1 exhibited $AUC_{0-\infty}$ values of 570.76, 565.87, and 472.17, respectively.

Antimicrobial susceptibility. Colistin, vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole MIC values are reported in Table 1. Colistin resistance had a minimal impact on the susceptibilities of other tested antimicrobials. The greatest impact observed was a 2-doubling reduction of the trimethoprim-sulfamethoxazole MIC value in the pairs of related *P. aeruginosa* isolates (from ≥128 μg/ml in *P. aeruginosa* ATCC 27853 to 32 μg/ml in PAm1). In contrast, vancomycin had no activity against any of the *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* isolates tested (MIC > 128 μg/ml), whether they were susceptible or resistant to colistin. Of note, *K. pneumoniae* ATCC 700603 and

TABLE 1 Antimicrobial susceptibility

Isolate	MIC (μg/ml)[a]			
	CST	VAN	TMP	TMP/SMX (1/19)
A. baumannii				
ATCC 19606	1	>128	32	16/304
ABm1	8	128	32	16/306
AB1	0.5	>128	64	8/152
AB2	1	>128	>128	>128/2,432
K. pneumoniae				
ATCC 700603	0.5	>128	4	2/38
KPm1	32	>128	4	2/38
KP3	0.5	>128	>128	>128/2,432
KP4	0.5	>128	>128	>128/2,432
P. aeruginosa				
ATCC 27853	0.5	>128	>128	>128/2,432
PAm1	8	>128	>128	32/612
PA4	0.5	128	>128	>128/2,432
PA5	1	128	>128	>128/2,432

[a] CST, colistin; VAN, vancomycin; TMP, trimethoprim; SMX, sulfamethoxazole.

KPm1 both displayed low MIC values for trimethoprim and trimethoprim-sulfamethoxazole (1/19) (4 and 2/38 μg/ml, respectively).

***In vitro* evaluation of antimicrobial combinations.** The checkerboard assay aimed to evaluate the potential for synergy of antimicrobial combinations at fixed concentrations. All tested combinations were synergistic ($0.18 < \Sigma FIC \leq 0.5$) against *A. baumannii* isolates. Interestingly, the ΣFIC values observed with the colistin-resistant derivative ABm1 were slightly lower than those observed with its parent susceptible strain, *A. baumannii* ATCC 19606 (0.18 versus 0.25, 0.375 versus 0.5, and 0.25 versus 0.37 for vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole, respectively). None of the combinations was synergistic ($\Sigma FIC > 0.5$) against any of the colistin-susceptible *K. pneumoniae* isolates or any of the *P. aeruginosa* isolates tested, including PAm1. In contrast, ΣFIC values of 0.2 to 0.25 were observed for all antimicrobial combinations against the colistin-resistant derivative KPm1 (Table 2).

The potential for synergy and bactericidal activity of each combination was further evaluated by static time-kill experiments at 0.25× and 0.5× MIC using inocula of 10^5 to 10^6 CFU/ml. Tested alone at 0.25× and 0.5× MIC, all the antimicrobials resulted in at least 1 $\log_{10}$ CFU/ml increase of the viable count at 24 h (data not shown). Changes observed at 24 h confirmed the results observed in the checkerboard assay against all *A. baumannii* isolates, i.e., the potential for synergy of colistin combined with vancomycin at 32 and 64 μg/ml (considered for the purposes of the assay to be 0.25× and 0.5× MIC), and with trimethoprim or trimethoprim-sulfamethoxazole (1/19) at 0.5× MIC (Table 2). The limit of detection (1 $\log_{10}$ CFU/ml) was achieved for most of the tested combinations demonstrating synergy (Table 2). Bactericidal activity (≥3 $\log_{10}$ CFU/ml) was achieved within 2 to 4 h against all *A. baumannii* isolates, but the killing activity of the combinations was slightly slower against ABm1 than *A. baumannii* ATCC 19606 (Fig. 2A and B).

None of the tested combinations demonstrated synergy against clinical isolates of colistin-susceptible *K. pneumoniae* (KP3 and KP4) using the time-kill assay (Table 2). Colistin plus trimethoprim or trimethoprim-sulfamethoxazole (1/19) demonstrated synergy at 0.5× MIC against *K. pneumoniae* ATCC 700603, and sustained bactericidal activity was achieved at 2 h. The limit of 3 $\log_{10}$ CFU/ml decrease in the viable count was achieved with colistin plus vancomycin at 0.5× MIC at 4 h, but sustained bacterial regrowth was observed afterward (Fig. 2C). In contrast and of interest, all tested combinations were synergistic and bactericidal against KPm1, with a time to achieve 99.9% kill

TABLE 2 *In vitro* evaluation of the bacteriostatic (checkerboard assay) and bactericidal activity (time-kill assay) of colistin combinations at 0.5× and 0.25× MIC[a]

Isolate	CST + VAN			CST + TMP			CST + TMP/SMX (1/19)		
	0.5× MIC	0.25× MIC	ΣFIC	0.5× MIC	0.25× MIC	ΣFIC	0.5× MIC	0.25× MIC	ΣFIC
A. baumannii									
ATCC 19606	−4.72 ± 0.05	−4.70 ± 0.03	0.25	−4.33 ± 0.33	+3.46 ± 0.36	0.50	−4.40 ± 0.27	+3.65 ± 0.60	0.37
ABm1	−4.74 ± 0.01	−4.61 ± 0.13	0.18	−4.83 ± 0.01	−0.71 ± 0.37	0.37	−4.23 ± 0.07	+2.15 ± 0.38	0.20
AB1	−4.54 ± 0.10	−1.26 ± 0.63	0.50	−4.25 ± 0.78	+3.03 ± 0.36	0.5	−4.65 ± 0.12	+3.05 ± 0.40	0.37
AB2	−4.88 ± 0.13	−4.74 ± 0.18	0.37	−2.66 ± 0.36	+3.42 ± 0.17	0.37	−2.04 ± 0.36	+3.21 ± 0.41	0.5
K. pneumoniae									
ATCC 700603	+2.20 ± 0.04	+2.93 ± 0.13	>0.50	−4.00 ± 0.41	+2.98 ± 0.38	>0.5	−3.00 ± 0.40	+3.57 ± 0.64	>0.5
KPm1	−3.01 ± 0.24	−1.47 ± 0.84	0.25	−4.67 ± 0.03	−1.99 ± 0.63	0.25	−4.82 ± 0.09	−3.85 ± 1.45	0.25
KP3	+3.23 ± 0.50	+3.26 ± 0.34	>0.50	+3.38 ± 0.60	+3.59 ± 0.69	>0.5	+2.84 ± 0.24	+3.32 ± 0.27	>0.5
KP4	+2.92 ± 0.15	+3.52 ± 0.25	>0.5	+2.76 ± 0.46	+3.36 ± 0.39	>0.5	+3.00 ± 0.13	+3.31 ± 0.18	>0.5
P. aeruginosa									
ATCC 27853	+3.98 ± 0.28	+3.52 ± 0.25	>0.5	+3.95 ± 0.46	+2.80 ± 0.01	>0.5	+3.95 ± 0.10	+3.31 ± 0.18	>0.5
PAm1	−1.65 ± 0.26	+3.05 ± 0.01	>0.5	−4.32 ± 0.32	+2.40 ± 0.50	>0.5	−4.82 ± 0.18	+2.26 ± 0.01	>0.5
PA4	+2.59 ± 0.50	+3.21 ± 0.04	>0.5	+2.66 ± 0.47	+2.77 ± 0.46	>0.5	+2.38 ± 0.73	+3.16 ± 0.14	>0.5
PA5	+3.58 ± 0.84	+4.06 ± 0.10	>0.5	+2.60 ± 0.30	+3.60 ± 0.60	>0.5	+2.86 ± 0.35	+4.01 ± 0.33	>0.5

[a] CST, colistin; VAN, vancomycin; TMP, trimethoprim; SMX, sulfamethoxazole.

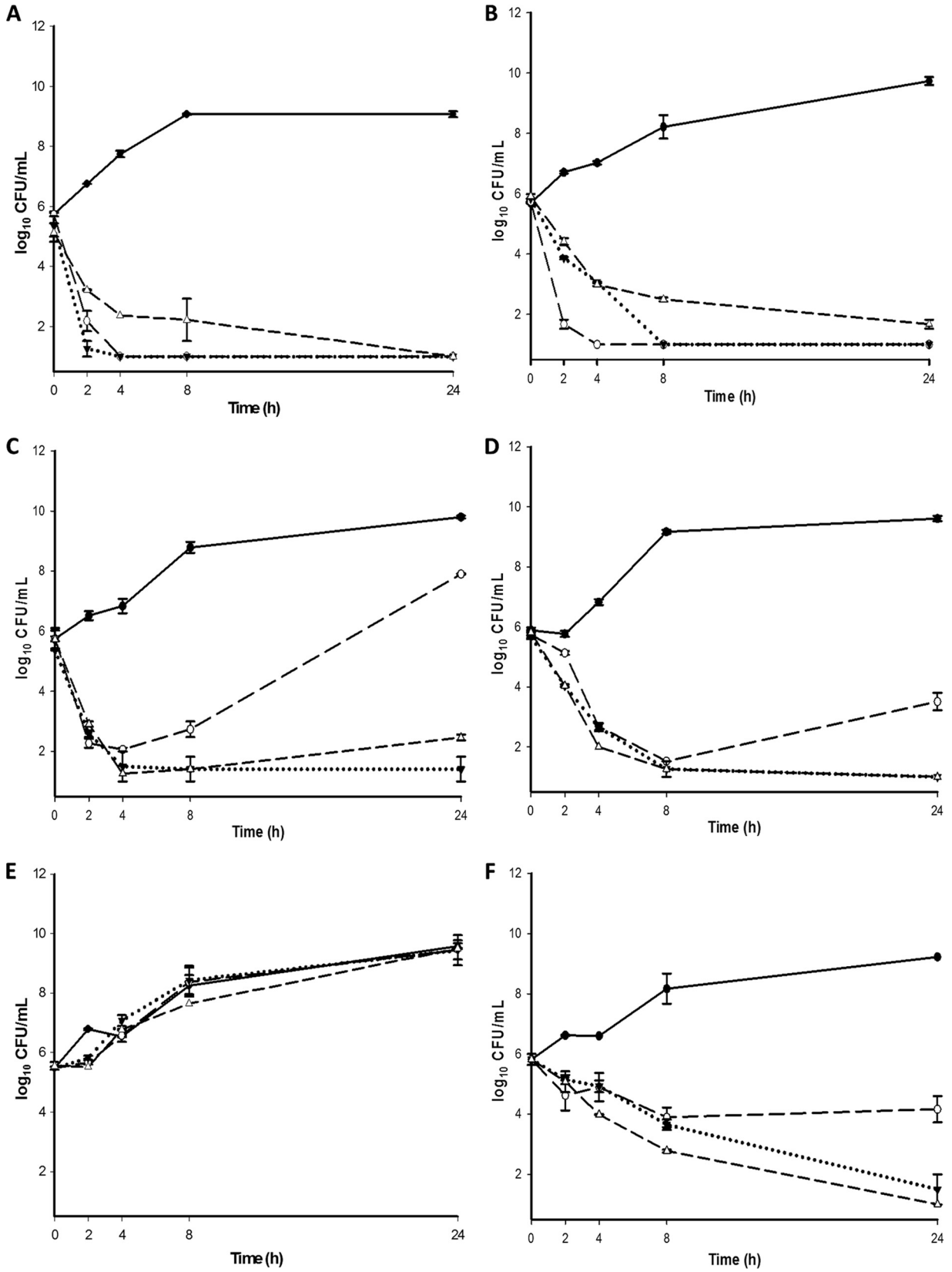

FIG 2 *In vitro* evaluation of the bactericidal activity of colistin combinations at 0.5× MIC against *A. baumannii* ATCC 19606 (A), *K. pneumoniae* ATCC 700603 (B), *P. aeruginosa* ATCC 27853 (C), ABm1 (D), KPm1 (E), and PAm1 (F). ●, growth control; ○, colistin plus vancomycin; ▼, colistin plus trimethoprim; △, colistin plus trimethoprim-sulfamethoxazole. The error bars indicate standard deviations.

($T_{99.9\%}$) of 4 h (Fig. 2D), and no bacterial regrowth was noted at 0.5× MIC (Fig. 2D).

None of the combinations demonstrated synergy at 0.25× and 0.5× MIC against the colistin-susceptible *P. aeruginosa* strains (*P. aeruginosa* ATCC 27853, PA4, and PA5) (Table 2 and Fig. 2E). In contrast, all combinations resulted in a greater than 2 $\log_{10}$ CFU/ml reduction at 0.5× MIC against PAm1 (Table 2). Colistin plus trimethoprim or trimethoprim-sulfamethoxazole (at 0.5× MIC) achieved sustained bactericidal activity against PAm1 at 8 h, whereas colistin plus vancomycin achieved a maximum of -1.90 ± 0.51 $\log_{10}$ CFU/ml at 8 h (Fig. 2F) and -1.65 ± 0.26 $\log_{10}$ CFU/ml at 24 h (Table 2).

DISCUSSION

Colistin's ability to disturb outer membrane permeability has been previously used to investigate the potential for synergy of colistin in combination with unconventional drugs, such as glycopeptides (13, 25), or hydrophobic compounds, such as trimethoprim (10). Thus, the synergy and bactericidal activity of colistin plus trimethoprim or vancomycin have been observed both *in vitro* and *in vivo* (in a *Galleria mellonella* model) against multidrug-resistant (MDR) *A. baumannii* isolates (13, 14, 25). However, data are available against only colistin-susceptible strains, and very little is known regarding the potential for synergy of colistin combinations against colistin-resistant Gram-negative bacteria. Bergen et al. have recently reported on the therapeutic potential of colistin plus doripenem against a single colistin-resistant clinical *P. aeruginosa* isolate using an *in vitro* pharmacokinetic/pharmacodynamic model (5). Li et al. also reported on the potential for synergy and substantial killing of colistin plus ciprofloxacin (-2.45 $\log_{10}$ CFU/ml) against an MDR *P. aeruginosa* strain resistant to colistin and ciprofloxacin (18). These preliminary results suggest that combinations of colistin with other antimicrobials may exhibit activity against colistin-resistant strains. To support this theory, Bergen et al. suggested that colistin-resistant strains have subpopulations that are heterogeneously resistant to colistin and that subpopulations for which the colistin MICs are higher may have the potential to be more susceptible to other antimicrobials (4). Our study aimed to evaluate the impact of reduced colistin susceptibility on the potential for synergy and the bactericidal activity of unconventional colistin combinations.

The collection of isolates tested included three pairs of colistin-susceptible/resistant *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* isolates and six clinical strains susceptible to colistin. Consistent with the literature (5, 17), the presence of a subpopulation heterogeneously resistant to colistin was observed for all tested isolates, regardless of the MIC value. However, colistin-resistant strains (MIC $>$ 4 μg/ml) exhibited higher proportions of subpopulations ($AUC_{0-\infty} > 472.17$) than their related susceptible strains ($AUC_{0-\infty} < 255$). These results suggest not only the high potential for selection of colistin-resistant subpopulations during therapy, but also the potential activity of unconventional antimicrobials and combinations, since increased susceptibility to a large panel of antimicrobial classes has been previously reported (16). The mechanisms behind the phenomenon of subpopulations heterogeneously resistant to colistin have been investigated in species such as *P. aeruginosa* and *A. baumannii*, but not much is known regarding species like *K. pneumoniae*. The roles of genes involved in membrane metabolism, such as the *pmr* genes encoding lipopolysaccharide modification enzymes or the *mexAB-oprM*, *mexCD-oprJ*, and *muxABC-opmB* genes encoding antimicrobial efflux transporters, have been demonstrated (1, 8), suggesting the potential clinical impact of this phenomenon in multidrug-resistant Gram-negative isolates. The mechanisms responsible for the emergence of subpopulations heterogeneously resistant to colistin in our study are under investigation in order to further understand the impact of colistin exposure on phenotypic and genotypic characteristics of major Gram-negative pathogens.

Also consistent with previously published data (13), we found that colistin plus vancomycin, trimethoprim, or trimethoprim-sulfamethoxazole (1/19) displayed synergistic and bactericidal effects against all colistin-susceptible *A. baumannii* isolates. The time to achieve 99.9% kill was found to be only slightly delayed in ABm1 compared to the colistin-susceptible isolates, suggesting that the alterations responsible for colistin resistance in *A. baumannii* isolates do not significantly affect the potential for synergy of the 3 unconventional combinations.

In contrast with the literature, no activity was observed for colistin-susceptible trimethoprim- and trimethoprim-sulfamethoxazole-resistant *K. pneumoniae* isolates, (KP3 and KP4). Indeed, combinations of colistin plus trimethoprim or trimethoprim-sulfamethoxazole have been previously demonstrated to be synergistic against various Gram-negative species, including *P. aeruginosa*. Exceptions were made, however, for a few isolates that were sulfamethoxazole resistant (21), which was true of KP3 and KP4.

Of particular interest in this study, synergy and bactericidal activities were demonstrated for colistin plus vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole at 0.25× and 0.5× MIC against KPm1, a colistin-resistant derivative of *K. pneumoniae* ATCC 700603. As observed and previously suggested by Bergen et al. (5), the presence of higher proportions of subpopulations heterogeneously resistant to colistin in KPm1 may favor the intracellular penetration and activity of the unconventional agents tested. Further investigations, including additional isolates and different experimental conditions, however, are warranted to better understand the impact of colistin and/or colistin resistance on the different components of antimicrobial resistance in *K. pneumoniae*.

Finally, and for the first time, we report on the activity and potential interest of colistin combinations against colistin-resistant strains of *P. aeruginosa*. Indeed, although no activity was observed with any of the tested combinations against the 3 tested colistin-susceptible *P. aeruginosa* strains, all the combinations showed synergy and/or bactericidal activity at 0.5× MIC against PAm1, the colistin-resistant derivative of *P. aeruginosa* ATCC 27853. Population analysis profiles of PA4, PA5, and *P. aeruginosa* ATCC 27853 revealed the presence of subpopulations heterogeneously resistant to colistin, but their proportions were significantly lower than for PAm1, which might explain why no synergy was observed in susceptible isolates. Further investigations, including extensive population analysis profiles and identification of markers of colistin resistance within the different species, are therefore warranted to better understand and estimate the clinical impact of our results.

Although our data suggest and somewhat confirm that colistin resistance is favorable to the *in vitro* activity of unconventional combinations, these results should be considered with care for several reasons. First, the antimicrobial concentrations required to observe synergy and bactericidal activities were well above clin-

ically relevant concentrations (up to 9, 40, 1.72, and 1.72/68 μg/ml with colistin, vancomycin, trimethoprim, and trimethoprim-sulfamethoxazole standard therapies at steady state). However, small ΣFIC values recorded for ABm1 (less than 0.30) may suggest that lower (and perhaps clinically relevant) concentrations might result in synergy and bactericidal activity, but this remains to be confirmed. Finally, extrapolation of these results to any other Gram-negative bacilli may not be appropriate at present, since the colistin-resistant strains used in the study were generated *in vitro* from laboratory strains, and the mechanism(s) by which colistin resistance occurs in PAm1, ABm1, and KPm1 remains unclear. Additional *in vitro* and *in vivo* studies using the same or different antimicrobial agents and colistin-resistant clinical strains are therefore warranted.

Finally, it now seems crucial to evaluate the clinical significance of these observations and to better understand the dose-response relationships of combinations like colistin plus trimethoprim-sulfamethoxazole. Unconventional combinations, such as vancomycin plus colistin, may not have any clinical value, considering the risk of nephrotoxicity of each agent and the concentrations required to obtain bactericidal activity, but additional *in vitro* studies may provide more evidence or insights into the mechanism of colistin resistance and how it may be favorable to antimicrobial combinations.

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C.V., L.B., and R.E.D. have no conflicts to declare.

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